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<b>(54) Title:</b> METHODS FOR PREVENTION OF CELLULAR PROLIFERATION AND RESTENOSIS  <b>(57) Abstract</b>  <p>Methods for the prevention and treatment of diseases involving cellular proliferation and dysfunctional apoptosis are provided. The methods involve administration of PKC inhibitors and derivatives. Additionally, local administration of protein kinase C inhibitors are provided for the treatment of restenosis and cancer therapies.</p>		

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## **METHODS FOR PREVENTION OF CELLULAR PROLIFERATION AND RESTENOSIS**

### **FIELD OF THE INVENTION**

The invention relates to a method for the prevention and treatment of diseases involving enhanced cellular proliferation and/or decreased cellular apoptosis, particularly smooth muscle cell proliferation leading to restenosis of blood vessels following revascularization intervention. The method comprises administration of protein kinase C inhibitors, particularly local administration of staurosporine derivatives. In a further aspect, the present invention relates to the use of protein kinase C inhibitors for the preparation of a medicament for the treatment or prevention of restenosis following revascularization.

### **BACKGROUND OF THE INVENTION**

Many individuals suffer from heart and peripheral cardiovascular disease caused by a partial blockage of the blood vessels that supply the heart and other organs with nutrients. Partial blockage often leads to hypertension, ischemic injury, stroke, or myocardial infarction. Atherosclerosis is a common form of biologically mediated vascular injury leading to stenosis. Additionally, mechanical injuries can also lead to vascular stenosis. Such mechanical injuries can often result following balloon angioplasty, vascular surgery, transplantation surgery and other similar invasive processes that disrupt vascular integrity. Consequently, arterial restenosis often occurs in human patients following revascularization intervention.

Percutaneous transluminal angioplasty is one well established revascularization intervention to reduce symptoms resulting from stenotic atherosclerotic plaques. Both angioplasty and coronary bypass surgery provide revascularization of stenotic arteries, particularly coronary arteries. Angioplasty offers a lower rate of procedure-related death or

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serious complications, as well as lower cost and reduced hospitalization time due to the minimally invasive nature of angioplasty.

Angioplasty of coronary arteries involves insertion of an angioplasty catheter in the femoral artery, threading the catheter through the vascular system to the site of the stenotic occlusion and dilation of the angioplasty balloon to crack open the stenotic plaque. Angioplasty of other arteries involves similar techniques with variations depending on the type of artery.

While angioplasty has a primary success rate of 90-95%, late restenosis or vessel reclosure, occurs in approximately 30-50% of patients within three to six months of the procedure. Late restenosis causes little increase in mortality, however it causes a substantial increase in morbidity in the form of angina, myocardial infarction, or need for repeat revascularization via angioplasty or bypass. More than half of the patients with angiographically defined restenosis require an additional revascularization procedure within six months and 80% with restenosis require repeat revascularization within six years.

Besides angioplasty, several other revascularization techniques are available to restore unimpeded blood flow to damaged blood vessels. These techniques include intraluminal stents, lasers and atherectomy devices. The problem of restenosis with these techniques is documented to be similar to those obtained with balloon angioplasty.

Therefore, restenosis has been identified as a key unmet medical need. Pharmacological treatment of restenosis has yet to be successful and local delivery of pharmacological agents has been suggested to "not be expected to yield substantial incremental benefit" (Lincoff et al., (1994) *Circulation* 90(4): 2070-2084). Systemic pharmacological treatment has a risk of toxicity associated with the high doses necessary for administration systemically to obtain adequate local pharmaceutical effect. This systemic intolerance of doses required to achieve local effect is a significant obstacle to the treatment of restenosis following revascularization. There are limited therapies available for its treatment or prevention. There is therefore a need for the prevention of restenosis following revascularization.

### **SUMMARY OF THE INVENTION**

Methods for the treatment or prevention of restenosis are provided. The methods involve administration of therapeutically effective amounts of protein kinase C (PKC) inhibitors, particularly staurosporine derivatives and antisense molecules. Additionally, local administration of PKC inhibitors is provided. The methods inhibit smooth muscle cell proliferation, stimulate apoptosis and inhibit stenosis following revascularization. In a further aspect, the present invention relates to the use of protein kinase C inhibitors for the preparation of a medicament for the treatment or prevention of restenosis following revascularization.

The compositions also find use as antineoplastic agents for the treatment of cancer, particularly in methods for local delivery.

### **DETAILED DESCRIPTION OF THE INVENTION**

The invention provides a method of preventing or treating restenosis in hollow tubes, increased cell proliferation or decreased apoptosis in a mammal in need thereof by administering a therapeutically effective amount of a composition comprising protein kinase C (PKC) inhibitors. Prevention of restenosis includes inhibition of restenosis so that it occurs at a lesser rate or results in less restenosis than would occur without treatment. The administration may be by one or more of the following routes: orally, parenterally, intravascularly, intranasally, intrabronchially, interperitoneally, esophageal, transdermally, or locally.

Restenosis includes the reocclusion of hollow tubes after mechanical dilation or ablation, particularly revascularization. Reocclusion can occur as a result of cellular proliferation or local deposition of cellular products. Hollow tubes include circulatory system vessels such as blood vessels (both arteries and veins), tissue lumen, lymphatic pathways, digestive tract including alimentary canal, respiratory tract, excretory system tubes, reproductive system tubes and ducts, body cavity tubes, tissue lumen, etc. Preferably, hollow tubes are circulatory

vessels. Dilation or ablation include the restoration of access to hollow tubes following an occlusion. Revascularization usually refers to the restoration of flow to circulatory vessels, particularly arteries following angioplasty or other physical intervention. Revascularization for purposes of this invention usually does not include regrowth and repair of circulatory tissue following injury such as bruising.

Local administration of the PKC inhibitors, including staurosporine derivatives and antisense molecules, are a preferred administration route. Local application of the drug affords concentrated delivery of the drug, achieving tissue levels in target tissues not otherwise obtainable through other administration routes. As drug release occurs via diffusion, a time-dependent process is involved and delivery to the target site will be sustained for days to weeks or beyond, depending upon the delivery system utilized. Further, local delivery reduces systemic drug exposure, thereby limiting systemic side effects. Local delivery allows for delivery agents which might otherwise be difficult or impossible to deliver via oral or intravenous routes due to problems of solubility or formulation. Local administration also provides the possibility of utilizing agents which might not otherwise be administratable because of concerns or toxicity encountered with conventional routes of administration.

Means for local drug delivery to hollow tubes can be by physical delivery of an active ingredient either internally or externally to the hollow tube. Local internal drug delivery includes balloon catheter delivery systems, endovascular polymer coated stents, facilitated diffusion, polymeric endoluminal paving, controlled release matrices, cell targeting such as affinity based delivery, receptor mediated cell toxicity, and the like. See, Eccleston et al. (1995) *Interventional Cardiology Monitor* 1:33-40-41 and Slepian, N.J. (1996) *Intervente. Cardiol.* 1:103-116, which disclosures are herein incorporated by reference. Local external drug delivery includes external patches around the hollow tube, hollow tube cuff, external paving, external stent sleeve, etc. Moreover, delivery could be by genetic therapy of appropriate tissue cells.

In one embodiment of the invention, biocompatible polymers are applied via catheter to the endoluminal surface following angioplasty. Polymers are applied to the surface of an organ or organ-component and custom-contoured *in situ* to yield a layer or film of polymer in intimate contact with the underlying tissue surface. Particularly preferred polymers include

polymeric hydrogels or colloidal systems which can be utilized as the applied polymeric material. Hydrogels are polymeric materials that are highly swollen but not dissolved by water. See, for example Slepian (1993) *Transactions of the Society for Biomaterials* XVI:233; Slepian *et al.* (1993) *Circulation* 88 :1-660 (abstract), Slepian *et al.* (1993) *Circulation* 33:1-9; and Hoffman DSN Kronenthal *et al.* (eds.) *Polymers in Medicine & Surgery*, New York: Plenum, pp. 33-44 (1974), which disclosures are herein incorporated by reference. Such endoluminal hydrogels function as local, direct contact, arterial wall drug delivery reservoirs. Additionally, the hydrogels provide drug delivery for a few days to several weeks following application. Furthermore, hydrogels readily allow incorporation and subsequent release of entrapped drugs, materials, or cells. Gel materials for use in the invention include poly(oxalkylene) polymers, *i.e.*, poly( $\epsilon$ -caprolactone), polyethylene glycol (PEG) - lactice polymers, etc.

It is recognized that drug diffusion can be regulated by the porosity of the hydrogel or the method by which the active ingredient is dispersed within the hydrogel. For more rapid drug diffusion, hydrogels of greater porosity can be utilized. In this manner, the length of drug delivery and treatment can be regulated by the porosity of the hydrogel. For prevention of restenosis, a slow release gel which delivers the compounds for at least about 2 days to about 1 year, preferably about 2 to about 30 days, more preferably about 8 to about 16 days, most preferably from about 10 to about 14 days is utilized.

In like manner, where the compounds of the invention are delivered locally to tumor cells, a slow release gel is used which delivers the compounds for at least about 2 days to about 1 year, preferably about 10 to about 30 days, more preferably about 15 to about 25 days

Means for delivery or application of the compounds in a slow release gel are available in the art. Several catheter systems have been designed for localized *in situ* formation of both thin and thick hydrogel paving layers. The catheter systems may vary depending upon the nature of the flowable baseline material utilized for hydrogel formation as well as the chemical or physical means utilized to locally convert the pre-gel constituents to a gel locally. See, for example, Slepian *et al.* (1993), *supra*, and Hill *et al.* (1993) *Circulation* 33:1-319, herein incorporated by reference.

Delivery or application of the compounds can also occur using stents or sleeves. An intraluminal stent composed of or coated with polymer or other materials into which the active drug has been impregnated can be used. Such stents can be biodegradable or can be made of metal or another stable substance when intended for permanent use. Also, luminal and/or abluminal coating or external sleeve made of polymer or other materials that contain the active drug can also be used for localized delivery.

A variety of PKC inhibitors are available in the art for use in the invention. These include bryostatin (U.S. Patent 4,560,774), safinogel (WO 9617603), fasudil (EP 187371), 7-hydroxystaurosporin (EP 137632B), various diones described in EP 657458, EP 657411 and WO9535294, phenylmethyl hexanamides as described in WO9517888, various indane containing benzamides as described in WO9530640, various pyrrolo [3,4-c]carbazoles as described in EP 695755, LY 333531 (IMSworld R & D Focus 960722, July 22, 1996 and Pharmaprojects Accession No. 24174), SPC-104065 (Pharmaprojects Accession No. 22568), P-10050 (Pharmaprojects Accession No. 22643), No. 4432 (Pharmaprojects Accession No. 23031), No. 4503 (Pharmaprojects Accession No. 23252), No. 4721 (Pharmaprojects Accession No. 23890), No. 4755 (Pharmaprojects Accession No. 24035), balanol (Pharmaprojects Accession No. 20376), K-7259 (Pharmaprojects Accession No. 16649), Protein kinase C inhib, Lilly (Pharmaprojects Accession No. 18006), and UCN-01 (Pharmaprojects Accession No. 11915). Also see, for example, Tamaoki and Nakano (1990) *Biotechnology* 8:732-735; Posada *et al.* (1989) *Cancer Commun.* 1:285-292; Sato *et al.* (1990) *Biochem Biophys. Res. Commun.* 173:1252-1257; Utz *et al.* (1994) *Int. J. Cancer* 57:104-110; Schwartz *et al.* (1993) *J. Natl. Cancer Inst.* 85:402-407; Meyer *et al.* (1989) *Int. J. Cancer* 43:851-856; Akinaga *et al.* (1991) *Cancer Res.* 51:4888-4892, which disclosures are herein incorporated by reference. Additionally, antisense molecules can be used as PKC inhibitors. Although such antisense molecules inhibit mRNA translation into the PKC protein, such antisense molecules are considered PKC inhibitors for purposes of this invention. Such antisense molecules against PKC inhibitors include those described in published PCT patent applications WO 93/19203, WO 95/03833 and WO 95/02069, herein incorporated by reference. Such inhibitors can be used in formulations for local delivery to prevent cellular



proliferation. Such inhibitors find particular use in local delivery for preventing tumor growth and restenosis.

PKC inhibitors of particular interest include staurosporine derivatives, particularly N-substituted staurosporine compounds, more particularly, N-benzoyl staurosporine. See, U.S. Patent No. 5,093,330, herein incorporated by reference. Other preferred inhibitors are the antisense molecules against PKC, preferably those as mentioned herein, particularly ISIS 3521 and ISIS 9606.

N-benzoyl staurosporine is a benzoyl derivative of the naturally occurring alkaloid staurosporine. It is chiral compound ( $[\alpha]_D^{20} = +148.0 \pm 2.0^\circ$ ) with the formula  $C_{35}H_{30}N_4O_4$  (molecular weight 570.65). It is a pale yellow amorphous powder which remains unchanged up to 220°C. The compound is very lipophilic ( $\log P > 5.48$ ) and almost insoluble in water (0.068 mg/l) but dissolves readily in DMSO.

Staurosporine derivatives inhibit a variety of protein kinase C isoforms which phosphorylate serine and threonine residues in a variety of target proteins critically involved in cell proliferation and differentiation. PKC enzymes have also been shown to regulate apoptosis (programmed cell death) and extra cellular matrix deposition. N-benzoyl staurosporine has additional activity against the platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) tyrosin kinases. Both PDGF and FGF are released at the time of angioplasty and both have potent affects on proliferation and matrix formation.

Preference is furthermore given to an antisense molecule according to the present invention which has a "chimeric" structure. The term "chimeric" structure with respect to antisense molecules is well known by those of skill and will therefore be explained in the following only for completeness. Within the context of the present invention, a "chimeric" structure, also termed a "chimera", is to be understood as meaning an antisense molecule (an oligonucleotide) which contains 2 or more chemically different regions which are in each case synthesized from one type of nucleic acid building block. Such chimeric antisense molecules typically comprise at least one region of modified nucleic acid building blocks which confer one or more advantageous property/properties (for example increased resistance to nucleases, increased binding affinity or diminished occurrence of sequence-independent side-effects) on

the antisense molecule, the so-called "wing", and a region which enables RNase H-mediated cleavage of the target nucleic acid to take place, i.e. the so-called "RNase H window". The affinity of an antisense molecule customarily determined by measuring the  $T_m$  value of the antisense molecule/target nucleic acid hybrid. The  $T_m$  value is the temperature at which the antisense molecule and the target nucleic acid dissociate from a previously formed hybrid. The dissociation is determined spectrophotometrically. The higher the  $T_m$  value, the higher is the affinity of the antisense molecule for the target nucleic acid. Methods for determining the  $T_m$  value belong to the state of the art (cf., for example, Fritsch and Maniatis, "Molecular Cloning - A Laboratory Manual", 2nd Edition, Cold Spring Harbor Laboratory Press, 1989). Within the context of the present invention, increased resistance to nucleases denotes decreased or slowed-down degradation of the oligonucleotide derivatives according to the invention by exonucleases or endonucleases which are present in a cell. The resistance to nucleases or the degradation of an antisense molecule can be monitored by gel electrophoresis, for example. RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of the enzyme therefore leads to cleavage of the target RNA and consequently increases the efficacy of the antisense mechanism. Cleavage of the target RNA can customarily be demonstrated by gel electrophoresis. Since, in a chimera, different advantageous properties are present in one and the same molecule, chimeric antisense molecules according to the invention possess a pronounced antisense effect with regard to inhibiting the expression of protein kinase C.

ISIS 9606 is a 20 mer chimeric phosphorothioate oligonucleotide targeting the 3'-untranslated region coding for human PKC- $\alpha$ . It is a chemically modified version (it contains 2'-O-methoxyethyl (= 2'-O-CH<sub>2</sub>CH<sub>2</sub>-OCH<sub>3</sub>) modifications at the 5'- and 3'-ends of the oligonucleotide (positions 1-6 and 15-19) flanking an eight base deoxynucleotide sequence (position 7-14)) of the phosphorothioate oligonucleotide ISIS 3521, that has been shown to specifically and potently reduce PKC- $\alpha$  mRNA and protein expression (Dean et al., (1994) *J. Biol Chem.* 269:16416-16424). The nucleotide base sequence of ISIS 9606 as well as of ISIS 3521 is

(5'-GTTCTCGCTGGTGAGTTTCA-3') (SEQ ID NO:1).

Accordingly, in one aspect the present invention relates to the use of a PKC inhibitor for the preparation of a medicament for the treatment or prevention of restenosis following revascularization. In a preferred embodiment thereof, the PKC inhibitor is a staurosporine derivative, in particular N-benzoyl staurosporine. In another preferred embodiment thereof, the PKC inhibitor is an antisense molecule. Preferably, such an antisense molecule has a nucleotide base sequence as set forth in SEQ:ID:NO. 1, like e.g. ISIS 3521. More preferably, the antisense molecule has a nucleotide base sequence as set forth in SEQ ID NO:1 and has a chimeric structure, like, in particular, ISIS 9606.

PKC inhibitors and staurosporine derivatives can be utilized to inhibit late restenotic lesion development in humans and other animals. For non-humans, early lesions are those which occur between 7 and 14 days post-injury. Usually, inhibition of smooth muscle cell (SMC) migration alone is sufficient to produce a reduction in early lesion size. Late lesions, in preclinical animal models such as rat, rabbit and pig, are lesions which occur more than 3 weeks post-injury (usually measured at 3, 4, 6, or 8 weeks). In humans, late lesions occur over a prolonged period of time compared to non-humans (usually 3-9 months post-injury). Compounds which only inhibit SMC migration usually do not inhibit late lesion size. In the present invention, inhibition of late lesions requires inhibition of intimal SMC proliferation (and possibly the stimulation of intimal SMC apoptosis, and/or inhibition of extracellular matrix and constrictive remodeling, as well). Post-injury for the present invention usually means revascularization intervention.

The compounds can be utilized to inhibit intimal smooth muscle proliferation, matrix formation and constrictive remodeling and stimulation of apoptosis.

The present invention is also drawn to local application of the compounds for their antitumor activity. Such local delivery of the compounds provides the benefits of local administration discussed above.

Solubility characteristics of N-benzoyl staurosporine appear to make it an ideal candidate for incorporation into a slow-release formulation for local delivery, particularly a slow release gel. The local delivery of the present invention allows for high concentration of compound at the disease site with extremely low concentration of circulating compound, obviating concerns

that systemic administration at high dosages may be associated with effects on proliferating tissue, such as in the intestine, testes and bone marrow.

It is recognized that the amount of compound used for local delivery applications will vary according to the indication being treated, etc. For purposes of the invention a therapeutically effective amount will be administered. By therapeutically effective amount is intended an amount sufficient to inhibit cellular proliferation and resulting in the prevention and treatment of the disease state. Specifically, for the prevention or treatment of restenosis after revascularization, local delivery gel formulation requires much less compound than systemic administration. For deposition in hollow tubes in general, and for deposition in human arteries for the treatment of restenosis in particular, about 200 to about 10,000 $\mu$ g is utilized, specifically about 400 to about 5000 $\mu$ g, more specifically about 500 to about 1000 $\mu$ g per deposition site. A deposition site is typically from about 10 to about 1000 mm squared in size. For antitumor activity, the compounds can be locally applied at rates of about 200 to about 10,000 $\mu$ g, specifically about 400 to about 5000 $\mu$ g, more specifically about 500 to about 1000 $\mu$ g per day for from about 2 days to about 1 year. When the active ingredient is an antisense molecule, the amount used is typically lower than when other PKC inhibitors such as staurosporine derivatives. Such amounts can be in the nM range, preferably from about 50 nM to 1000 nM, more preferably concentrations of at least 200 nM.

The compounds of the invention may be used individually or alternatively more than one compound can be formulated into the gel or other local delivery device for delivery.

### EXAMPLES

The following examples are offered by way of illustration and not by way of limitation.

#### Example 1

##### Characterization of N-benzoyl Staurosporine

N-benzoyl staurosporine (CGP 41251) is a highly selective inhibitor of protein kinase C ( $IC_{50}$  = 50 nM) and has demonstrated antiproliferation properties in vitro ( $IC_{50}$  = 100 nM) making it a potentially ideal candidate for local application. This drug's high partition

coefficient ( $\log P > 5.48$ ) and near insolubility in aqueous media (.068 mg/l) may lead to improved tissue retention and reduced loss to blood flow following local deposition in the blood vessel. N-benzoyl staurosporine has demonstrated extended stability in physiologic buffers (pH 7-9). The compound has the ability to produce a micronized form ( $< 5 \mu\text{m}$ ). Therefore, for purposes of the invention, the compound can be used as a particulate suspension or encapsulation to control release (slow dissolution or diffusion) from a hydrogel polymer.

Thermal stability ( $T_g \approx 200^\circ\text{C}$ , decomposition at  $320^\circ\text{C}$ ) and organic solubility (methylene chloride, 1,2 propylene glycol) of N-benzoyl staurosporine provide flexibility in preparing formulations for use in the present invention. Solubility in polyethylene glycol (PEG) improves the changes for successful incorporation within a gel polymer, a PEG based material. Analytical methods for detection of active substance and metabolites have been developed. [ $^{14}\text{C}$ ] Staurosporine derivatives are available in sufficient quantity for gel formulation release kinetics study.

#### Example 2

##### In Vitro Inhibition of Proliferation and Stimulation of Apoptosis of Vascular Smooth Muscle

**Cell Culture:** Rat aortic A10 SMC were purchased from American Type Culture Collection (Rockville, MD). Human coronary arteries were purchased from Clonetics Corp. (San Diego, California). Primary porcine SMC were isolated from the coronary artery of porcine hearts from small pigs (55-77 kg) which were obtained from a local abattoir. The left anterior descending artery and left circumflex were dissected from each heart and excess muscle tissue cut away. The vessels were opened longitudinally and the endothelial layer was scraped away with a cotton-tipped applicator. The intimal layer of SMC was peeled away and incubated, with magnetic stirring, in an enzyme solution containing collagenase (460 U/ml), papain (25 U/ml) and DL-dithiothreitol (0.4 mg/ml) in Dulbecco's phosphate-buffered saline for 55 minutes at  $37^\circ\text{C}$ . The supernatant was discarded and the pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 20% FBS, 5% penicillin-streptomycin and the resulting flocculent mass was shaken to release the cells. Any remaining debris was removed by filtration through a fine mesh. Cells were plated in  $25 \text{ cm}^2$  Falcon Primaria flasks at a density of  $2 \times 10^6$  cells. Cells were allowed to grow for 5 days before

medium was removed and replaced with DMEM containing 10% FBS, 1% penicillin-streptomycin. Cultures were identified as SMC by their characteristic hills-and valleys growth pattern and immunofluorescence with anti- $\alpha$  smooth muscle actin monoclonal antibody.

Rat (passages 18-21) and porcine SMC (passage 2-6) were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 1% glutamine and 1% penicillin-streptomycin at 37°C in a 95% air/5% CO<sub>2</sub> humidified atmosphere. Human SMC (passage 3-7) were grown in Smooth Muscle Cell Growth Media (SMGM)(Clonetics Corp., San Diego California) supplemented with 0.5  $\mu$ g/ml hEGF, 5  $\mu$ g/ml insulin, 1  $\mu$ g/ml hFGF, 5% FBS and 50  $\mu$ g/ml gentamycin at 37°C in a 95% air/5% CO<sub>2</sub> humidified atmosphere. Cells were subcultured by aspiration of the growth medium followed by a 30-sec rinse with a solution of 0.5 mM EDTA/0.05% trypsin.

**Measurement of Proliferation:** SMC, grown in 96-well microtiter plates (5,000 cells/well) to 80% confluence, were washed twice with phosphate-buffered saline and growth-arrested by replacing the medium with DMEM supplemented with 0.2% FBS. On the day of the experiment, the medium was removed and replaced with DMEM (without phenol red) containing 10% FBS for rat and pig SMC or SMGM (without phenol red) containing 5% FBS for human SMC. Compounds were dissolved in DMSO, at 10 mM, and serial dilutions of drug were made into culture media. Cells were incubated with PKC inhibitors (10  $\mu$ l) for 15 minutes prior to the addition of FBS (10  $\mu$ l) in a final volume of 100  $\mu$ l. Rat and porcine VSMC (vascular SMC) were treated for 24 and 72 hours, respectively, with increasing concentrations of PKC inhibitors. Proliferation was measured using the colorimetric CellTiter Cell Proliferation Assay (Promega Corp.) which measures the reduction, by living cells only, of the tetrazolium compound 3-zolium compound (MTS) to formazan. Briefly, 20  $\mu$ l of MTS was added to each well during the last 3 hours of stimulation with mitogen and the plates returned to the 37°C incubator. The absorbance of formazan at 490 nm was recorded using a microplate reader (Molecular Devices Corporation).

**Measurement of DNA Fragmentation:** VSMC, grown in 96-well microtiter plates (5,000 cells/well) to 80% confluence were labeled overnight with 5-bromo-2'-deoxyuridine (10  $\mu$ M) at 37°C. After labeling, the cells were centrifuged (10 min, 250 g) and resuspended into

culture medium. Cells were incubated with PKC inhibitors for varying time periods. Cells were then lysed, fragmented DNA was separated from intact chromatin by centrifugation, and internucleosomal DNA fragmentation was quantitatively determined using a DNA fragmentation photometric ELISA kit (Boehringer Mannheim). The absorbance at 30 nm is recorded using a microplate reader (Molecular Devices Corporation).

Alternatively, DNA fragmentation was assessed quantitatively by DNA agarose gel electrophoresis. Cells were removed from 75cm<sup>2</sup> flasks by gentle scraping after the indicated treatments. The cell suspension was transferred by a conical centrifuge tube, and the cells were pelleted by centrifugation at 750 x g for 5 min. The supernatant was removed, and the cells were resuspended in 5ml PBS.

**Results:** N-benzoyl staurosporine (CGP 41251) potently inhibited serum-induced proliferation in cultured rat, porcine and human vascular smooth muscle cells with corresponding IC<sub>50</sub> values of 303, 78, 100 nM, respectively, whereas the inactive analog CGP 42700, was without effect. Staurosporine demonstrated antiproliferative IC<sub>50</sub> values of 34, 7 and 0.8 nM in serum-stimulated rat, porcine, and human smooth muscle cells, respectively. Treatment of rat smooth muscle cells with staurosporine of CGP 41251 resulted in a time-dependent releases of DNA fragments into the cytoplasm, as measured by photomeric ELISA and DNA agarose electrophoresis, indicating that these compounds induce apoptosis in these cells.

### Example 3

#### In Vivo Inhibition of Restenosis in the Rat Carotid Balloon-Injury Model

CGP 41251 was tested in the rat carotid balloon-injury model of restenosis.

**Animals:** Male Sprague Dawley rats weighing 350 to 500 gm were housed individually and allowed to acclimate prior to surgery. All animals received standard rat chow and water ad libitum. Group size was normally 12 animals per group.

**Dosing:** CGP 41251 or analogues were administered per os (p.o.) once per day, either as a suspension of active ingredient in cornstarch or, in the case of CGP 41251, gelucire formulation suspended in distilled water. Doses of the compound varied per experiment and ranged between 25 mg/kg/day and 100 mg/kg/day of active ingredient. In all but the "window

therapy" studies, compound was administered once daily 5 days prior to surgery and daily post-balloon injury throughout the entire study. Study length varied from 4 to 56 days post-balloon-injury. For "window therapy" studies, compound was given once daily 5 days pre-ballooning and daily post-ballooning for time courses ranging from 5 to 14 days post-ballooning. In all studies, control animals were dosed with appropriate vehicle using the same dosing schedule as compound-treated animals.

For local delivery studies using analogues of CGP 41251, such as CGP 75139A, compound administration was perivascular. A segment of ballooned carotid was encircled with a 1 cm length of silastic tubing (0.25 inch inside diameter, .047 inch outside diameter) to which was attached a catheter which feeds into an osmotic pump containing either compound or vehicle. This delivery system provided continuous, local delivery to the adventitia of the wrapped portion of vessel. Local compound administration ranges between 5 ug and 10 mg, locally, per day, depending on the solubility characteristics of the individual compounds.

**Balloon Injury Surgery:** The left common carotid arteries were denuded of endothelium using a 2F Fogarty catheter as previously described (Prescott *Am. J. Pathol.* (1991) 139:1291-1296; Clowes et al., (1983) *Lab Invest.* 49:327-333; Baumgartner, (1963) *Z Ges. Exp. Med.* 137:227-249). Briefly, rats were anesthetized with ketamine (50 mg/ml) and rompun (10 mg/ml) administered intraperitoneally at a dose of 1.5 ml/kg. A midline incision was made in the neck to expose the left external and common carotid arteries. The balloon was inserted into the common carotid artery via the left external branch, inflated with saline, and pulled back three times through the lumen with a rotating motion to ensure maximal endothelial denudation. The catheter was then removed, the external carotid artery was ligated and the wound was closed. Each animal was given an injection of the antibiotic Bacillin (200,000 units/kg) and the analgesic Buprenorphine (0.06 mg/kg) immediately following surgery.

**Termination, Histology and Morphometry:** Animals were killed at the following time periods ranging between 4 and 56 days post-balloon injury. One half hour before termination, blood was collected, centrifuged, and stored at -20 degrees Centigrade for analysis of circulating levels of compound. 5% Evans Blue was then injected intravenously to allow discrimination of re-endothelialized areas at the time of histologic processing.



Animals were killed by administration of an overdose of ketamine and rompun. The carotid arteries were pressure-perfusion fixed using STF (Streck Labs) at 100 mmHg. For local delivery studies, the osmotic pumps were recovered and the volume of remaining content was recorded to ensure that pump failure had not occurred.

Carotid arteries were excised and immersion fixed in STF for 1-5 days, then transferred to Ringer's solution. Two samples from control blue region of each left carotid artery were imbedded in paraffin. A minimum of six carotid sections, 20  $\mu$ M apart were cut per animal and stained with Verhoff Elastic stain to produce a modified Verhoff stain. Intimal and medial area measurements were performed with a digitizing tablet interfaced to a VIDAS imaging system. Any sections in which disruption of the internal elastic lamina was observed were excluded from measurement. Additional cross sections were cut from 3 week and 6 week animals and stained with Verhoff-Van Geisen to allow visual determination of the extent of connective tissue deposition.

**Measurement of SMC Migration:** Since SMC migration into the intima is known to first occur between days 3 and 4 post ballooning, the number of intimal smooth muscle cells at 4 days post injury was determined as previously described (Prescott et al., (1991) *Am. J. Pathol.* 139: 1291-1296). Briefly, the number of smooth muscle cells either within the intima or observed to be migrating through the internal elastic lamina was counted in a minimum of 4 cross sections per animal. Cross sectional analysis was considered to be preferable to an en face technique because it allowed the exclusion of animals in which the internal elastic lamina was observed to have been disrupted.

**Measurement of Neointimal SMC Proliferation:** An additional 4 cross sections were cut from carotids obtained from animals killed at 7 and 9 days post injury. Sections were immunostained using monoclonal antibodies against proliferating cell nuclear antigen. Briefly, sections of rat ileum served as positive controls. Nuclei in the proliferation phase of the cell cycle were identified as dark-staining nuclei. More than half of the nuclear contour had to be darkly stained in order to be considered a PCNA-positive nucleus. The number of PCNA-positive cells within the neointima was counted and expressed as a percentage of the total number of neointimal cells counted on a Verhoff-stained serial section.

**Results:** Table 1 shows the results of various PKC inhibitors and the effect on early and late restenosis after balloon angioplasty. 50 mg/kg p.o. reduced both lesion size and intimal SMC proliferation at 9 days (d) post-injury (70% and 43%, respectively). Late lesions at 3 weeks were inhibited dose-dependently (25, 50, and 100 mg/kg/d inhibited 26%, 43% and 66% respectively). This inhibition of late lesions was demonstrated to be unique; numerous compounds including matrix metalloprotease (MMP) inhibitors, angiotensin converting enzyme (ACE) inhibitors, angiotensin II antagonists, calcium channel blockers and heparin inhibit early but not late lesion formation in this model.

Table 1

PKC Inhibitor (CGC 41251)	<u>7-10 days post ballooning</u>	<u>21-28 post ballooning</u>
50 mg/kg p.o. (in cornstarch)	70% lesion size decrease 43% decrease in neointimal SMC proliferation	
25 mg/kg p.o. (in gelucire)		26% lesion size decrease
50 mg/kg p.o. (in gelucire)	57% lesion size decrease	43% lesion size decrease
100 mg/kg p.o. (in gelucire)		66% lesion size decrease
50 mg/kg (in gelucire) (window therapy treatment 5 days post ballooning)		8% lesion size decrease
50 mg/kg (in gelucire) (window therapy		25% lesion size decrease

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treatment 10 days

post ballooning)

50 mg/kg (in gelucire)

40% lesion size decrease

(window therapy

48% lesion size decrease

treatment 14 days

post ballooning)

Efficacy of CGP 41241 on late lesions appears due to its combined ability to reduce intimal SMC division, inhibit matrix deposition, and stimulate SMC apoptosis. Constrictive remodeling may also be reduced as external elastic lamina perimeters were larger in treated animals. To support local delivery activities, studies were performed to determine the minimum number of days of CGP 41251 therapy needed to reduce late lesions. Therapy for the first 10 days post-angioplasty reduced 4-week lesions by 24% ( $p < .05$ ), 14 days of therapy reduced late lesions to a similar extent as daily treatment for the full 4 weeks (43%,  $p < .01$ ). Using a perivascular cuff attached to a continuous delivery pump (Alza), staurosporine analogues can be delivered locally to the vessel wall. These experiments show that local administration of staurosporine derivatives inhibit intimal lesions similar to systemic administration.

Increased smooth muscle cell proliferation and matrix formation play key roles in development of late restenotic lesions. In addition, apoptosis appears to be downregulated in restenotic lesions. Preclinical studies in animal models of restenosis showed N-benzoyl staurosporine inhibited late restenotic lesion development. This effect appears to be due to its effect on several signal transduction pathways, resulting in potent inhibition of intimal smooth muscle cell proliferation, matrix formation and stimulation of apoptosis. Preclinical studies also demonstrated that 14 days of treatment postangioplasty reduces late lesion formation to a similar extent as that achieved by 28 days of treatment.

#### Example 4

##### Reduced Toxicity of Local versus Systemic Delivery

Dogs and rats were given CGC 41251 p.o. for extended periods of time. Tissue samples from the animals were analyzed for toxicological effects. The no adverse effect level in a three month toxicity study was 10 mg/kg in the rat and 1 mg/kg in the dog and in the 6-month (interim) study in the rat was 3 mg/kg. Treatment with CGP 41251 at doses of and above 3 mg/kg in dogs (up to 3 months) and rats (up to 6 months) was associated with effects on

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proliferating tissues. The most sensitive tissues were the intestine, testes and bone marrow. Effects on bone marrow were accompanied by hematological changes. Treatment of rats with CGP 41251 was consistently associated with alterations of liver function parameters (e.g., ALAT, ASAT). However, these effects were not accompanied by pathological changes and may not be significant. In dogs, inhibition of spermatogenesis was seen at all doses (3, 10 and 30 mg/kg) after 3 months treatment in the initial study but not at 3, 1, or 0.3 mg/kg in a repeat study in male dogs. At the 3 mg/kg level, the systemic exposure of the dogs to CGP 41251 in the repeat study, in which no toxic effects were seen, was probably as high as that observed in the initial study. The difference in the findings is, therefore, most probably a reflection of the sensitivity of the individual animals. From the results of the repeat 3 month study, it was concluded that the no effect level for 41251 was 3 mg/kg. However, in view of the testicular effects seen in the previous three month study at this dose, it was concluded that 1 mg/kg represents a clear no effect level in the male beagle dog after repeated oral administration for 3 months.

#### Example 5

##### Inhibition of PKC Inhibitors with Antisense Molecules

Human lung carcinoma cells are grown and treated with 200 nM of an antisense molecule such as ISIS 9606. PKC mRNA levels are measured as described Dean and McKay (1994) *Proc. Natl. Acad. Sci. USA* 91:11762-11766. PKC mRNA levels are reduced and cell division is decreased. When the same experiments are conducted using SMC cultures, PKC mRNA levels are reduced. Administration of ISIS 9606 in the in vivo model of Example 3 shows both reduced lesion size and Intimal SMC proliferation.

The methods of the present invention offer advantages not currently available in the art. Local application of drugs via a gel polymer offers therapeutic advantage not provided by systemic administration or physical anti restenotic devices, e.g. metal stenting. The gel-drug polymer functions as a synthetically interposed tissue barrier. Thus, following revascularization, restenosis, matrix formation, smooth muscle cell proliferation and

dysfunctional apoptosis is prevented and/or treated as well as the prevention of the adhesion of platelets to the surface.

All publications and patent applications mentioned in the specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes for clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

- (A) NAME: Novartis AG
- (B) STREET: Schwarzwaldallee 215
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- (E) COUNTRY: Switzerland
- (F) POSTAL CODE (ZIP): 4058
- (G) TELEPHONE: +41 61 324 1111
- (H) TELEFAX: +41 61 322 7532

(ii) TITLE OF INVENTION: Methods for prevention of cellular proliferation and restenosis

(iii) NUMBER OF SEQUENCES: 1

## (iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "oligonucleotide"



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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GTTCCTCGCTG GTGAGTTTCA

20

**CLAIMS****WHAT IS CLAIMED IS:**

1. A method for the treatment or prevention of restenosis following revascularization said method comprising administering a therapeutically effective amount of a PKC inhibitor for a time sufficient to prevent restenosis.
2. The method of claim 1, wherein the PKC inhibitor is a staurosporine derivative.
3. The method of claim 2, wherein the staurosporine derivative is N-benzoyl staurosporine.
4. The method of claim 3, wherein the N-benzoyl staurosporine is administered systemically.
5. The method of claim 3, wherein the N-benzoyl staurosporine is administered locally.
6. The method of claim 5, wherein the local administration comprises a polymeric hydrogel.
7. The method of claim 6, wherein the hydrogel comprises a poly(oxyalkylene) polymer.
8. A method for the treatment or prevention of restenosis following revascularization, said method comprising the local delivery of at least one protein kinase C inhibitor.
9. The method of claim 8, wherein the revascularization is balloon angioplasty.
10. The method of claim 8, wherein the protein kinase C inhibitor is a staurosporine derivative.
11. The method of claim 9, wherein the protein kinase C inhibitor is N-benzoyl staurosporine.
12. The method of claim 11, wherein the local administration comprises a polymeric hydrogel.
13. The method of claim 12, wherein the hydrogel comprises a poly(oxyalkylene) polymer.
14. A method for preventing or alleviating smooth muscle cell proliferation in a patient in need thereof, said method comprising administering locally an effective therapeutic amount of a protein kinase C inhibitor.

15. The method of claim 14, wherein administration is in an artery.
16. The method of claim 15, wherein administration is in a coronary artery.
17. The method of claim 15, wherein the method also inhibits lesion formation following revascularization.
18. The method of claim 17, wherein the revascularization is by balloon angioplasty.
19. The method of claim 17, wherein the protein kinase C inhibitor is a staurosporine derivative.
20. The method of claim 19, wherein the staurosporine derivative is N-benzoyl staurosporine.
21. The method of claim 20, wherein the local administration is a polymeric hydrogel.
22. The method of claim 21, wherein the hydrogel comprises a poly(oxyalkylene) polymer.
23. The method of claim 1, wherein the PKC inhibitor is an antisense molecule.
24. The method of claim 1 wherein the antisense molecule has a nucleotide base sequence as set forth in SEQ ID NO:1.
25. The method of claim 24 wherein the antisense molecule has a chimeric structure.
26. The method of claim 25, wherein the antisense molecule is ISIS 9606.
27. The method of claim 23, wherein the antisense molecule is administered locally.
28. The method of claim 27, wherein the local administration comprises a polymeric hydrogel.
29. The method of claim 28, wherein the hydrogel comprises a poly(oxyalkylene) polymer.
30. Use of a PKC inhibitor for the preparation of a medicament for the treatment or prevention of restenosis following revascularization.
31. The use according to claim 30, wherein the PKC inhibitor is a staurosporine derivative.
32. The use according to claim 31, wherein the staurosporine derivative is N-benzoyl staurosporine.

33. The use according to claim 30, wherein the PKC inhibitor is an antisense molecule.
34. The use according to claim 33, wherein the antisense molecule has a nucleotide base sequence as set forth in SEQ ID NO:1.
35. The use according to claim 34, wherein the antisense molecule is a chimeric oligonucleotide.
36. The use according to claim 35, wherein the antisense molecule is ISIS 9606.

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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>A61K 31/55, 31/70, 48/00, 45/08</b>		<b>A3</b>	(11) International Publication Number: <b>WO 98/07415</b> (43) International Publication Date: 26 February 1998 (26.02.98)
(21) International Application Number: PCT/EP97/04503 (22) International Filing Date: 18 August 1997 (18.08.97) (30) Priority Data: 60/024,219 20 August 1996 (20.08.96) US 60/025,072 30 August 1996 (30.08.96) US (71) Applicant (for all designated States except US): NOVARTIS AG [CH/CH]; Schwarzwaldallee 215, CH-4058 Basel (CH). (72) Inventor; and (75) Inventor/Applicant (for US only): PRESCOTT, Margaret, Forney [US/US]; 2 Fairview Road, Millburn, NJ 07041 (US). (74) Agent: ROTH, Bernhard, M.; Novartis AG, Patent- und Markenabteilung, Lichtstrasse 35, CH-4002 Basel (CH).			(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  Published With international search report. (88) Date of publication of the international search report: 7 May 1998 (07.05.98)
(54) Title: METHODS FOR PREVENTION OF CELLULAR PROLIFERATION AND RESTENOSIS			
(57) Abstract  Methods for the prevention and treatment of diseases involving cellular proliferation and dysfunctional apoptosis are provided. The methods involve administration of PKC inhibitors and derivatives. Additionally, local administration of protein kinase C inhibitors are provided for the treatment of restenosis and cancer therapies.			

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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/EP 97/04503

## A. CLASSIFICATION OF SUBJECT MATTER

A 61 K 31/55, A 61 K 31/70, A 61 K 48/00, A 61 K 45/08

According to International Patent Classification (IPC) or to both national classification and IPC 6

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A 61 K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database WPIL on Questel, week 9542, London: Derwent Publications Ltd., AN 95-325481, Class A 61 K; & JP,A,7-223958 (ASAHI KASEI KOGYO KK et al.), abstract.	1-4, 8-10, 30,31
X	Database WPIL on Questel, week 9337, London: Derwent Publications Ltd., AN 93-295063, Class A 61 M; & US,A,5242397 A (CEDARS SINAI MEDICAL CENT.), abstract.	1,2, 8-10, 14-19, 30,31
X	Database WPIL on Questel, week 9624, London: Derwent Publications Ltd.,	1-4, 8-10, 30,31

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

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Date of the actual completion of the international search  
15 December 1997

Date of mailing of the international search report  
20.02.98

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## INTERNATIONAL SEARCH REPORT

-2-

International Application No

PCT/EP 97/04503

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	AN 96-239258, Class A 61 K; & WO,A2,96/13259 (COR. THERAPEUTICS INC.), abstract. --	
X	Database WPIL on Questel, week 9319, London: Derwent Publications Ltd., AN 93-154410, Class A 61 K; & EP,A1,0541486 (CIBA GEIGY AG), abstract. --	1,8, 10,14- 16,30, 31
X	US 5093330 A (CARVATTI G. et al.) 03 March 1992 (03.03.92), abstract, claims 1,8, column 2, lines 7-29, example 4 (cited in the application). --	1-4, 30-32
X	EP 0588762 A1 (CIBA-GEIGY AG) 23 March 1994 (23.03.94), abstract, claims 1-14, page 3, line 39 - page 4, line 6, page 4, line 47 - page 5, line 8. --	1,8, 14,30
X	Database WPIL on Questel, week 9336, London: Derwent Publications Ltd., AN 93-288106, Class A 61 K, & WO,A1,93/16703 (SMITHKLINE BEECHAM CORP.), abstract. --	1,30
X	Database WPIL on Questel, week 9520, London: Derwent Publications Ltd., AN 95-155201, Class A 61 K; & WO,A1,95/09852 (CIBA GEIGY AG. et al.), abstract. --	1,30
X	Database WPIL on Questel, week 9020, London: Derwent Publications Ltd., AN 90-149206, Class A 61 K; & EP,A,0368223 (HOECHST AG), abstract. --	1,30
X	Database WPIL on Questel, --	1-3,



# INTERNATIONAL SEARCH REPORT

International Application No.

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

PCT/EP 97/04503  
Relevant to claim No.

Category \*

Citation of document, with indication, where appropriate, of the relevant passages

A

week 9342, London:  
Derwent Publications Ltd.,  
AN 93-336831, Class A 61 K;  
& WO,A1,93/20101 (GLAXO SA),  
abstract.  
--  
WO 93/19203 A1  
(ISIS PHARMACEUTICALS, INC.)  
30 September 1993 (30.09.93),  
abstract, claims 1,2,4,5,7,  
15-25,32,33, page 2, line 5 -  
page 3, line 10, table 1,  
example 5, fig. 1, page 12,  
lines 7-18, page 15,  
lines 3-8, page 28,  
point (2).  
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30,33

1,8,  
14,23,  
24,30,  
33,34

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP 97/04503

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1-29  
because they relate to subject matter not required to be searched by this Authority, namely:  
  

Remark: Although claims 1-29 are directed to a method of treatment of the human or animal body by therapy (Rule 39.1 (iv)PCT) the search has been carried out and is based on the alleged effects of the compounds.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## ANHANG

zum internationalen Recherchen-  
bericht über die internationale  
Patentanmeldung Nr.

## ANNEX

to the International Search  
Report to the International Patent  
Application No.

## ANNEXE

au rapport de recherche inter-  
national relatif à la demande de brevet  
international n°

PCT/EP 97/04503 SAE 170736

In diesem Anhang sind die Mitglieder  
der Patentfamilien der in obenge-  
nannten internationalen Recherchenbericht  
angeführten Patentdokumente angegeben.  
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This Annex lists the patent family  
members relating to the patent documents  
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In Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
JP A2 7223958	22-08-95	keine - none - rien	
US A 5242397	07-09-93	US A 5112305 HU B1 212760	12-05-92 28-02-97
WO A2 9613259	09-05-96	AU A1 427897/96 CA AA 2203757 EP A2 7883356 WO A3 9613259 US A 5574892	23-05-96 09-05-96 13-08-97 01-08-96 07-10-97
EP A1 541486	12-05-93	AU A1 281387/92 CA AA 20882216 FI A0 924983 FI A 924983 IL A0 1033660 JP A2 3222082 MX A1 9206408 NO A0 924284 NO A 924284 US A 9244431 ZA A 9206562	13-05-92 08-05-93 04-11-92 08-05-92 04-04-92 31-08-92 01-10-92 06-11-92 10-05-92 23-11-92 07-05-93
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